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EVALUATION OF EFFECT OF TROLOX ON ANTIOXIDANT STATUS IN STREPTOZOTOCIN INDUCED DIABETIC RATS

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ABSTRACT

Trolox is the white solid, cell permeable, water soluble derivative of vitamin E. The aim of this study was to evaluate the antioxidant effect of trolox on antioxidant status in streptozotocin induced diabetic rats. All albino wistar rats were divided into four groups and each group contained six animals. Group I (Normal control) received vehicle, group II (Diabetic control) received STZ (50 mg/kg i.p.). Group III and IV received STZ (50 mg/kg i.p.) along with trolox 10 mg/kg and 20 mg/kg respectively. All the groups were treated for seven days. On 8th day the blood was collected by retro orbital sinus punctured method and centrifuged at 3000 rpm for 10 min to separate the serum which was used for the determination of antioxidant enzymes, lipid peroxidation and uric acid. The result shows that antioxidant enzyme level such as SOD, CAT and Peroxidase in treated diabetic rats by trolox were significantly increased. The lipid peroxidation and uric acid level in serum from diabetic rats were significantly decreased by trolox. It was concluded that the administration of trolox in diabetic rats can exert beneficial effects of anti oxidative defense system against that imposed by diabetes mellitus.

Keywords: STZ , SOD,CAT, Trolox.

INTRODUCTION

Oxidative stress is in general as excess formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS include free radical such as superoxide (O_2), hydroxyl (OH), peroxyl (RO2) hydroperoxyl (HRO_2) as well as non radical species such as hydrogen peroxide (H_2O_2) and hydrochlorous (HOCL)RNS include free radicles like nitric oxide ($^{\cdot}NO$) and nitrogen oxide($^{\cdot}NO_2$) as well as non radicles such as peroxy nitrite ($ONOO^-$), nitrous oxide(HNO_2) and alkyl peroxynitrite (RONOO) of these reactive molecule,$^{\cdot}O_2$, $^{\cdot}NO$ and $^{\cdot}ONOO^-$ are the most widely studied species and play important role in the Diabetic complication. Direct evidence of oxidative stress in diabetes is based on studies that focused on the measurement of the oxidative stress markers such as urinary F2-isoprostane and plasma as well as plasma and tissue level of nitrotyrosine and $^{\cdot}O_2$ .There are multiple sources of oxidative stress in diabetes including non enzymatic, enzymatic and mitochondrial pathway.

Non enzymatic sources of oxidative stress originated from the oxidative biochemistry of glucose. Hyperglycemia directly caused the increase in ROS generation. Glucose can undergo auto oxidation and generate $^{\cdot}OH$ radicals. In addition glucose reacts with protein in non enzymatic manner leading to the development of the Amadori product followed by the formation of the AGE_s.

Enzymatic sources of the augmented generation of reactive species in diabetes include NOS, NAD(P)H oxidase and xanthine oxidase. All isoforms of NOS require 5 co factor groups such as flavin adenine dinucleotide (FAD), flavin mononucleotide(FMN), heme, BH_4, ca^{2+} - calmodulin.

The mitochondrial respiratory chain is the another source of non enzymatic generation of reactive species [1].

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Diabetes and Oxidative stress

The human body is exposed to free radicles from outside the body (exogeneous) and inside the body (endogenous).Some of the factors that lead to free radicals are smog, cigarette smoking, radiation, consumption of excessive amount of alcohol, and even sun light. Yet some of the factors that lead to free radicle come from within the body. The cells require oxygen to produce the energy that need to work properly. In the process known as mitochondrial respiration, the cells take in oxygen, burn it and release energy. During this process free radicles are produced. Oxidative stress occurs when the free radical exceeds the ability of body to neutralise them. This imbalance is happen because of two reasons

a) When the anti oxidant production is decreased ,
b) When the free radicles are produced in excess.

For instance diabetes, or the aging process itself, can direct to increased speed of the production of these endogeneous free radicals and reduced antioxidant resistance.

Oxidative stress functions on both sides, meaning that help the progression and development of diabetes and its complications. Oxidative stress is one of the important mediators of the vascular complications in diabetes including nephropathy. High glucose produces reactive oxygen species as a result of the glucose auto oxidation, metabolism and the development of the advanced glycosylation end product. Oxidative stress takes place while oxygen free radicles are produced in a very large amount through the diminution of oxygen. The result in diabetic children with type I diabetes showed extensively decreased glutathione peroxidase and plasma antioxidant capacity and increased in MTD that are increased in compare with the healthy child. In type 2 diabetes, free radicals production has reported to be increased in the diabetic patient and to be implicated in the development of diabetes complication [2].

POSSIBLE DIABETIC COMPLICATION

People with diabetes are most likely to get the diseases such as stroke, heart disease etc. Study it was shown that stroke is a serious complication of diabetes but the risk factors for stroke in the patient are not fully defined. The incidence of diabetes among patient with congestive heart failure (CHF) is increased. Mortality remains about 30% higher for the diabetics with CHF than non-diabetics. Multiple mechanisms are responsible for the development of CHF in diabetes with ischemic heart diseases and its attended complication of left ventricular dysfunction playing a major role [2].

Oxidative stress makes damaged in tissue or organ caused by free radicals. Reactive oxygen species (ROS) have been reported to be caused by chemicals such as streptozotocin (STZ) in experimental animals [3].

Trolox is an of white solid, cell permeable, water soluble derivative of vitamin E with potent antioxidant properties. It prevents peroxynitrite-mediated oxidative stress and apoptosis in rat thymocytes [4].

MATERIALS AND METHODS

Chemicals

Streptozotocin, Standard Malondialdehyde and Trolox were procured from the Sigma Aldrich, St. Louis, USA, Nitroblue Tetrazolium, Methionine, EDTA, Riboflavin, Guaicol, Trichloro acetic acid , Thiobarbituric acid and all other analytical reagents were of analytical grade.

Animals

Wistar albino rats weighing (170-200g) were maintained in identical laboratory condition and fed with commercial pellet diet (Hindustan Lever Kolkata, India) and water *ad libitum*. All procedures described were reviewed and approved by the IAEC, Anuradha College of Pharmacy, Chikhli. Dist.– Buldhana (Maharashatra).

Induction of experimental diabetes

Injection of freshly prepared solution of STZ (50 mg/kg i.p.) in 0.1 ml citrate buffer, pH 4.5 intraperitoneally in a volume of 1 ml/kg was used for the induction of diabetes. After 48 hrs of STZ administration, rats with moderate diabetes having hyperglycemia (i.e., with blood glucose level 200-300 mg/dl) were used for the experiment [5].

Determination of the blood glucose level

Blood samples were collected from the retro orbital sinus punctured method after 48 hrs of administration of STZ. Serum glucose concentration was determined using a clinical glucose diagnostic kit, based on glucose oxidase method [6].

Experimental procedure

Albino wistar rats were divided into four groups and each group contained six animals. Group I(Normal control) received vehicle, group II (Diabetic control) received STZ(50 mg/kg i.p).Group III and IV received STZ (50 mg/kg i.p.) along with trolox 10 mg/kg and 20 mg/kg respectively. All the groups were treated for seven days. On 8^{th} day the blood was collected by retro orbital sinus punctured method and centrifuged at 3000 rpm for 10 min to separate the serum which was used for the determination of antioxidant enzymes, lipid peroxidation, and uric acid [5].

Superoxide dismutase (SOD)

The method was based on the ability of Superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide.

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue

tetrazolin (NBT). The 3ml reaction mixture contain 50 mM phosphate buffer, pH7-8,13mM methionine, 75µm NBT, 2um riboflavin, 0-1 EDTA, and 10-100 µL of enzyme extract. Riboflavin was added last and the tubes were shaken and placed below the 30 cm below a light bank consisting of two 15 W florescent lamp. The reaction was started from by switching on the light and was allowed to run for 10 min which time it was found earlier to be linear. The reaction was stopped by switching off the light and the tubes were covered with the black cloths. The absorbance by the reaction mixture at 560 nm was read. A non–irradicate reaction mixture did not develop colour and serves as control. There is no measurable effect of the diffused room light. The reaction mixture lacking enzyme developed the maximum colour and this decreased with increasing volume of enzyme extract is added. The activity was calculated by following formula

$$U/ml = \frac{\Delta A \text{ min/ml} \times \text{test volume} \times \text{dilution factor}}{\text{Abs. Coefficient} \times \text{path length} \times \text{enzyme volume}}$$

Where ΔA min/ml is the time in min required to increase in A_{560} for approximately 5 minute [7].

Catalase CAT)

Assay based on disappearance of peroxide is followed spectrophotometrically at 240 nm. One unit decomposes one micromole of H_2O_2 per minute at 25°C and pH 7.0 under the specified condition. A 3 ml of reaction mixture was prepared by adding 50mM Potassium phosphate buffer (pH 7.0), 25µl of serum and 15mM hydrogen peroxide and measured the absorbance at 240 nm against the blank without hydrogen peroxide, at every 30sec. up to 3 min. The activity was calculated using following formula.

$$U/ml = \frac{3.45 \times Dr}{\text{Time in minute} \times 0.1}$$

Peroxidase

This method describe by Sadashivam and Manickam (1996) in which 3.0 ml of reaction mixture was taken in the cuvette containing 100mM of potassium phosphate buffer (pH 7.0), 0.05 ml of 20mM guicol solution, 0.1 ml of serum and 0.03 ml of 12.3mM hydrogen peroxide (H_2o_2). The reaction was mixed well and ongoing reaction was read at every 30 sec at 436 nm up to 3 minute against the blank without enzyme. The activity of the enzyme was calculated using the following formula:

$$U/ml = \frac{\Delta A \text{ min/ml} \times \text{test volume} \times \text{dilution factor}}{\text{Abs.Coefficient} \times \text{path lenth} \times \text{enzyme volume}}$$

Where ΔA min/ml is the time in min required to increase in A_{560} for approximately 5 min.

Uric acid

Serum uric acid concentration (mg/dl) was determined using a Clinical Uric Acid Diagnostic Kit, Based on the Biuret method.

Lipid Peroxidation/MDA (LPO)

To 0.5 ml serum, 2.5 ml of 20 mg/dl trichloacetic acid was added and the tube was left to stand for 10 min at room temperature. After centrifugation at 3500 rev./min for 10 min, the supernatant was decanted and the precipitate was washed once with 0.05 mM sodium ACD. Then 2.5 ml of 0.05 M sulphuric acid and 3.0 ml of 0.2 mg/dl TBA in 2 M sodium sulfate are added to this precipitate and the coupling of lipid peroxide with TBA was carried out by the heating in a boiling water bath for 30 min. After cooling in cold water, the resulting chromogen was extracted with 4.0 ml of n-butyl alcohol by vigorous shaking. Separation of the organic phase was fascilated by centrifugation at 3000 rev/min for 10 min and its absorbance was determined at the wavelength of 530 nm [8].

RESULTS AND DISCUSSION

The antioxidant activity of trolox was studied on streptozotocin induced diabetic rats by its ability to reduce the level of Lipid peroxidation (MDA) and the uric acid. The Antioxidant enzyme level such as SOD, CAT, and Peroxidase in treated diabetic rats by trolox was significantly increased. This study represent the effect of trolox at two dose level (10 mg/kg and 20 mg/ kg) on anti oxidant status on STZ induced diabetic rats.

STZ (50 mg/kg i.p.) was administered to all groups except group I rats. After 48 hrs blood was withdrawn to estimate blood glucose level. Blood glucose more than 200 mg/dl was considered as diabetic animals. Trolox at two dose levels (10 mg/kg and 20 mg/kg) was administered to group III and IV. Group II was considered as untreated diabetic rats (Toxicant control).On the 8th day blood was withdrawn to estimate blood glucose, antioxidant enzymes (SOD,CAT and Peroxidase), Lipid peroxidation and uric acid levels.

Reduced activities of SOD and CAT in serum have been observed during diabetes.SOD is an important enzyme which catalyses the dismutation of superoxide radicals.CAT is the heme protein which catalyses the reduction of hydrogen peroxides and protects the tissue from the highly reactive hydroxyl radicals. Therefore reduction in the activity of these enzyme (SOD,CAT) may result in the number of deleterious effect due to the accumulation of superoxide radicals and hydrogen peroxide [8]. Administration of trolox (10 mg/kg and 20mg/kg i.p.) for seven days significantly increased in the reduced level of SOD and CAT in diabetic rats compared with untreated diabetic rats.

The activity of peroxidase was observed to decrease significantly in diabetic rats. Peroxidase an enzyme catalyses the reduction of hydrogen peroxide and hydro peroxide to the non toxic products. The depletion in the activity of these enzymes may result in the involvement of deleterious oxidative changes due to the accumulation of toxic products [3].

Lipid peroxidation is one of the characteristic features of diabetes. Lipid peroxidase mediated damage has been observed in the development of both type I and II diabetes mellitus. It has been observed that insulin secretion is closely associated with the lipooxygenase derived peroxides .Low levels of lipooxygenase derived peroxides stimulate the secretion of insulin, but when the concentration of the endogeneous peroxides increases it may initiate uncontrolled lipid peroxidation leading to cellular infiltration and islet cell damage in type I diabetes mellitus. The observed significant increase in the level of blood glucose could be due to destruction of pancreatic β cell by STZ [9]. In our present study treated diabetic rats by trolox (10 mg/kg and 20 mg/kg) for seven days could significantly decreased lipid peroxidation as compared to diabetic control group.

Uric acid is considered as one of non enzymatic antioxidant. But increased production of uric acid lead to increased the production of free radical. This is due to activation of the xanthine oxidase enzyme system. In our experiment uric acid level is increased in diabetic rats. Trolox decreased the uric acid level.

Trolox is one of the most important drugs having a potent free radical scavenger activity. Trolox treated diabetic rats elevated reduced SOD, CAT and peroxidase activity and decrease elevated MDA and uric acid. It is evident that trolox gives protection against formation of free radicals. Trolox does not have a blood glucose lowering effect significantly.

Table 1. Effect of trolox on antioxidant status in STZ induced diabetic rats

Parameters	Normal Control	Diabetic Control	Trolox (10 mg/kg)	Trolox (20 mg/kg)
SOD (U/ml)	5.74±0.19	2.95 ± 0.06	3.48 ± 0.06	3.92 ± 0.6
CAT (U/ml)	20.66 ± 1.35	14.01 ± 0.13	24.19 ± 1.00	25.47 ± 0.99
Peroxidase (U/ml)	42.8 ± 1.61	28.9 ± 2.01	35.32 ± 1.43	38.4 ± 1.78
MDA (nmol/ml)	5.29 ± 0.01	6.94 ± 0.52	5.96 ± 0.03	5.68 ± 0.10
Uric acid (mg/dl)	2.32 ± 0.02	2.98 ± 0.05	2.27 ± 0.15	2.16 ± 0.02

Results were expressed as Mean±Sem. Statistical analysis was carried out by one way ANOVA followed by Dunnett's Multiple Comparison Test.

CONCLUSION

It is evident from the present study that trolox administration in diabetic rats may help in the prevention and protection against the formation of free radicals in the diabetes. The antioxidant effects of trolox in STZ induced diabetic rats were significant and trolox caused dramatic elevation of antioxidant systems activity along with decreased in lipid peroxidation and uric acid thus reduced oxidative stress. This result indicates the administration of trolox in diabetic rats can exert beneficial effects of anti oxidative defense system against that imposed by diabetes mellitus.

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